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Isolation and Identification of Psychrotrophic Photosynthetic Bacterium from Antarctic Seawater with Antibacterial Activity

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Abstract: The search for new antibiotics is on the rise because of the growing threat of antibiotic resistance. Once the antibiotics fail to work, people are at risk of dying of common infections, and routine operations become difficult to perform, making millions of lives at risk. In our study, the authors found that a photosynthetic bacterium can provide a good potential source of another group of antimicrobial compounds known as photoantimicrobial compounds. This psychrotrophic bacterium was isolated from the Antarctic and grew well at a temperature ranging from 10 °C to 30 °C in the acetate medium, implying minimum energy usage by the bacteria contributing to energy saving for its bioprocessing. The bacteria identified as *Stenotrophomonas* sp. via 16S rDNA analysis and coded as SZB2 strain was rod shaped. Gram negative and possessed bacteriochlorophyll *a* and *b* are usually found in photosynthetic bacteria. SZB2 was found to produce antibacterial substances from the cocultivation assay with both Gram negative and Gram positive bacteria such as *Escherichia coli* and *Staphylococcus aureus*. The growth inhibition was higher in *S. aureus* than that of *E. coli*. Hence, it may be concluded the bacteria had the ability to produce a wide spectrum antibiotic.

Key words: Antarctic, antibacterial activity, bacteriochlorophyll, photosynthetic bacteria.

1. Introduction

Several studies have documented that Antarctic regions represent extreme cold environments surrounded by a limited diversity of plants and animals but highly diverse microbial diversity [1] that are specifically adapted to constant low temperatures [2]. It has been demonstrated that the Antarctic also has a high frequency of pigment production in ice cores, glaciers, or marine surface waters [3]. Therefore, lack of microbial studies in the Antarctic makes it a valuable place for all types of scientific research [4]. In the Vestfold Hills of East Antarctica, purple and green sulfur bacteria inhabit a series of hypersaline lakes [5].

In recent years, there has been an increasing interest in studying photosynthetic bacteria due to their wide distribution in the environment, ability to carry out photosynthesis by using light as an energy source [6] and contain single cell proteins, carotenoid, biopolymers, bacteriochlorophyll, pantothenic acid and antimicrobial agents [7]. Photosynthetic bacteria are important for a wide range of scientific and industrial processes.

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Photosynthetic bacteria have a pivotal role in cosmetic, food industry, medicine [8] and wastewater treatment [9]. Photosynthetic bacteria have mechanisms for detoxification of toxic substances in wastewater that enable them to survive in toxic ridden wastewater [10]. There is a growing body of literature that recognizes the importance of photosynthetic bacteria in bioremediation, bioplastic and biohydrogen production [11]. Photosynthetic bacteria were mostly observed in soils, paddy fields, lakes, rivers, oceans, activated sludge, and extreme environments [12]. A number of researchers have reported that photosynthetic bacteria were able to grow as heterotroph or autotroph [13] under anaerobic or aerobic condition. Photosynthetic bacteria were divided into green and purple photosynthetic bacteria that convert light energy into chemical energy by photosynthesis in the presence of photosynthetic pigments such as bacteriochlorophyll and carotenoid [9].

Recently, there has been renewed interest in producing new antimicrobials due to the increasing cases of antimicrobial resistant bacteria. Photosynthetic bacteria are of interest because they can be a good potential source of new antimicrobial compounds which have been reported to have lower antimicrobial resistance [14]. Photosynthetic bacteria can play an important role in addressing the issue of rising antimicrobial resistance bacteria. The increase of antimicrobial resistant bacteria is increasingly recognized as a serious, worldwide public health concern. Apart from that, bacteria that exhibit antibacterial activities had been isolated from various water samples.

Surprisingly, antibacterial activity of the photosynthetic bacteria isolated from the Antarctic has not been empirically studied to the best of our knowledge. This study set out to isolate photosynthetic bacteria from Antarctic seawater samples and to identify the antibacterial activity of the photosynthetic bacteria isolated from Antarctic seawater samples. This research sheds new light on identification of potential antibacterial activity of photosynthetic bacteria from the Antarctic marine water sample. This paper attempts to contribute to this growing area of research by isolating and characterizing photosynthetic bacteria from the Antarctic that are particularly important in providing new insights into the diversity of these organisms in the Antarctic.

2. Materials and Method

2.1 Isolation and Characterization of Photosynthetic Bacteria

For the isolation of photosynthetic bacteria, the seawater samples from the Schollaert Channel, Antarctic were inoculated and grown under continuous illumination with approximately 300 lux light intensity in acetate [15] and succinate medium [16] at 10, 20, and 30 °C. Each liquid medium was adjusted to pH 6.8-7.2. The cultures were incubated until a light or dark red coloration was attained and OD (optical density) 600 nm measurements were performed to monitor the growth of the cultures in each medium at different temperatures. Single colonies were purified and obtained using the dilution streak method. Selected isolated bacteria were monitored for growth in acetate medium at 30 °C under light source at 600 nm at regular time intervals. The morphology of the isolates was examined based on the colony appearance, pigmentation, form, elevation, and margin. Gram staining was performed according to the procedure modified by Azhar et al. [17].

2.2 Photosynthetic Pigment Analysis

Photosynthetic bacteria were first detected by examining their absorption spectrum for the presence of photosynthetic pigment such as bacteriochlorophyll *a* and *b*, and carotenoid within a range of 200-1,100 nm utilizing the UV-Vis Spectrophotometer (Hach DR 600, Hach, U.S.A.).

2.3 Antibacterial Activity Analysis

The antibacterial activity of the isolated bacteria was determined using microbes including *Escherichia coli* (Gram negative bacteria) and *Staphylococcus aureus* (Gram positive bacteria) as the target microorganisms. The isolated bacteria were used to inoculate *E. coli* and *S. aureus* at different inoculation ratios of 5%, 10%, 15%, 20%, and 25% (*v*/*v*). This method is particularly useful in identifying the effectiveness of photosynthetic bacteria as an inoculum to inhibit the growth of *E. coli* and *S. aureus*. The culture was incubated for one week at 30 °C. Finally, after one week, the growth of the bacteria was monitored and recorded at 600 nm.

2.4 16S rDNA Sequence Analysis

Genomic DNA of the isolated bacteria was extracted using Genomic DNA Purification Kit (Promega), according to the manufacturer's protocol. After genomic extraction, the 16S rDNA gene was amplified with universal primer (27F and 1492R) [10] using Mastercycler® PCR (polymerase chain reaction) thermal cycler (Eppendorf, Hamburg, Germany) with temperature cycle as follows: initial denaturation at 94 °C for 4 min. Then 30 cycles were set of denaturation at 94 °C for 1 min, annealing at 48 °C for 30 s and extension at 68 °C for 2 min, followed by final extension at 68 °C for 5 min. After PCR cycles were completed, PCR products were run on gel electrophoresis by using 80 V for 45 min to ensure the presence of DNA. The PCR products were sent for purification and sequencing to First Base Laboratories, Malaysia. The obtained sequences were queried against the GenBank and Ribosomal Database Project using the Blast algorithms. Phylogenetic relationships were analyzed by the evolutionary distance matrix calculated using the neighbour-joining method. Evolutionary analyses were conducted in MEGA6 and editing of phylogenetic trees was done using Figtree Figure Drawing Tool Version 1.4.2.

3. Results and Discussions

In order to identify the optimum temperature for growth of the bacteria in Antarctic water samples, they were cultured at three different temperatures (10 °C, 20 °C, 30 °C). The bacteria isolated from the Antarctic could be classified as psychrotrophic since they grew well at temperature ranging from 10 to 30 °C in organic media. In general, the bacteria showed better growth in acetate medium especially at 30 °C (Fig. 1). The cells in the liquid medium did not produce any colour pigment at 10 °C. Meanwhile, a dark red pigment was produced at 20 °C and a light red pigment was produced at 30 °C. Prior studies have noted that the colour of photosynthetic bacterial pigments obtained in the cell suspension could vary from brown, red, pink, beige, to purple [9]. The most striking result to emerge from this data is that 30 °C was the optimum temperature for the growth of these bacteria. Interestingly, the bacteria were considered as psychrotroph even though they originate from Antarctica in a low temperature environment. This is an interesting result implying minimum energy usage by the bacteria contributing to saving energy for its bioprocessing.

In this study, growth was detected by observing the turbidity of the enrichment medium or in other words, the medium appeared cloudy. The turbidity can be measured in absorbance units using the UV-Vis spectrophotometer at wavelength 600 nm [6]. Red pigmentation of the culture medium was also observed

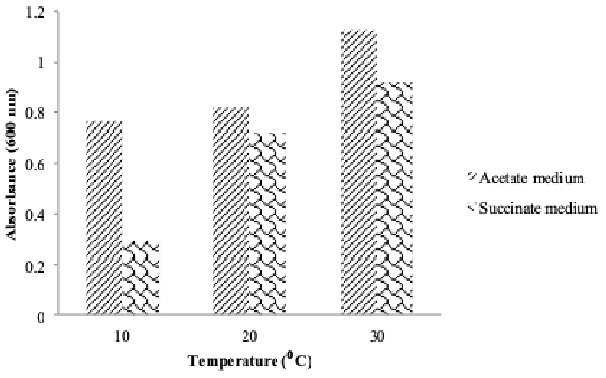

Fig. 1 Growth of bacteria in Antarctic water sample in a different medium at different temperature.

which implied the presence of photosynthetic bacteria [16]. The cultures, 200 μL, were then spread onto the succinate and acetate agar plates to obtain pure bacterial colonies using the spread plate method. They were then incubated at 30 °C in the presence of light until growth of bacterial colonies was observed. The bacteria were subsequently transferred onto succinate and acetate agar plates until pure colonies were obtained. A total of ten bacteria were isolated. Out of ten bacteria, only one bacteria that was named as SZB2 with the fastest growth was selected for further study.

The selected bacteria, SZB2 showed a typical growth profile of bacteria (Fig. 2). The bacteria had a long lag phase which lasted for more than 10 h. The exponential phase of SZB2 lasted for about 5 h. SZB2 experienced the death phase after more than 20 h of incubation. The specific growth rate was 0.075 h^{-1} and the generation time was 9.24 h.

As for the colony morphology which can also be used to identify and characterize the bacterial culture, when grown on the acetate agar plate, the colonies appeared white, circular form, with raised elevation and entire margin features. However, SZB2 only produced red colour pigment when grown in acetate broth but not in the acetate nutrient agar plate. In the agar plate, it was observed as white colonies. Several reports have shown that photosynthetic bacteria can also appear as colourless or white-coloured colonies [1].

Besides the colony morphology, SZB2 was examined for its cellular characteristics. Light microscope (Olympus C-35 AD-4) was utilized for the microscopic observation of the stained cells of SZB2. The Gram staining test showed that SZB2 was Gram negative bacteria and the cellular morphology was rods (data not shown). In general, Gram positive bacteria have thicker cell walls, containing mainly peptidoglycan that is able to retain the purple colour of crystal violet while Gram negative bacteria will appear pink after counterstaining with Safranin [17].

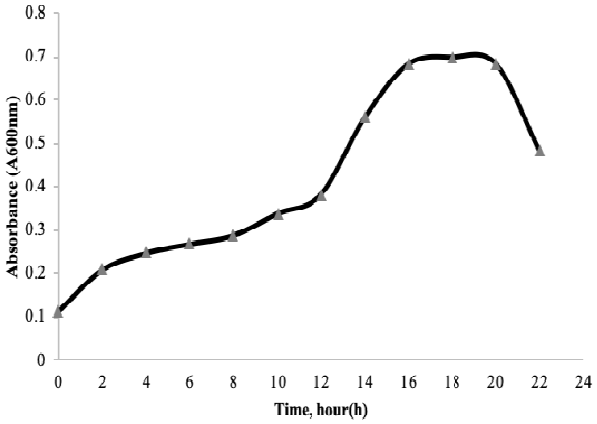

Fig. 2 Growth profile of bacteria SZB2.

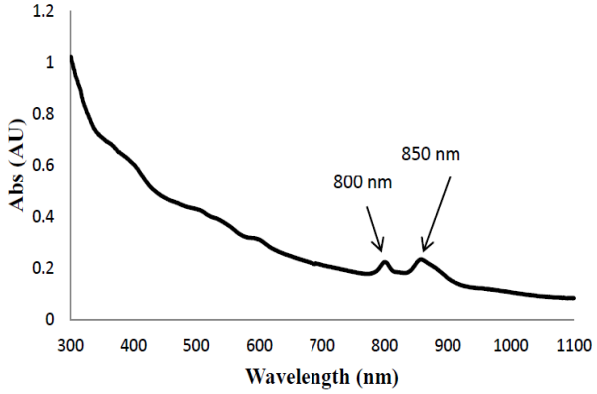

Fig. 3 *In-vivo* spectrum of the cell (photosynthetic pigment analysis of SZB2).

Photosynthetic pigment analysis was carried out to screen for the presence of pigment in SZB2. The cell suspension of SZB2 isolate was red with maximum absorption spectra at 800 and 850 nm (Fig. 3). Strong evidence of photosynthetic bacteria was found when SZB2 showed two main peaks at 800 and 850 nm which corresponded to bacteriochlorophyll *a* and *b* [6]. Bacteriochlorophyll contains more saturated tetrapyrrole ring than chlorophyll which caused bacteriochlorophyll to be detected at significantly longer wavelengths than chlorophyll [18]. Photosynthetic bacteria become purple (or a variety of other colours) due to carotenoids that are responsible for light harvesting and provide the main pigmentation in the visible region of the spectrum [18]. It was also reported, anoxygenic phototrophic purple bacteria absorb light via carotenoids at 500 nm and exceeding 800 nm via bacteriochlorophylls [19]. Various colours

of pigments from brown, red, pink, beige, purple appeared in the cell suspension of photosynthetic bacteria [9]. Previous studies had reported that photosynthetic bacteria could carry out photosynthesis without releasing oxygen gas to the cytoplasmic membrane and internal membrane systems [9] of photosynthetic bacteria that contain *in-vivo* pigment molecules (bacteriochlorophyll and carotenoid) which are non-covalently bound to proteins to form well organized pigment-protein complexes [19].

From antibacterial activity analysis, SZB2 was more effective towards the inhibition of *E. coli* growth at the inoculum concentration of SZB2 less than 25%. What is interesting in the data is that, at inoculum concentration of more than 25%, SZB2 was effective to both bacteria, *E. coli* and *S. aureus*. At inoculum concentration of 25%, the growth of *E. coli* and *S. aureus* was reduced to 69% and 66% respectively. This was the highest growth inhibition for *E. coli* and *S. aureus* in this study. Meanwhile, there is 100% growth for both bacteria (*E. coli* and *S. aureus*) when SZB2 was not added to the culture (these acted as controls). In Fig. 4, there is a clear trend of decreasing in percent growth of *E. coli* and *S. aureus*. With successive increase in inoculum concentration SZB2, the percent growth of *E. coli* and *S. aureus* showed growth was further inhibited. This experiment was repeated three times and the same trend in the results was obtained; the results were consistent with those obtained in the previous experiment. *E. coli* is a Gram negative bacterium that has a thinner cell wall as compared to *S. aureus.* Gram-negative bacteria are surrounded by a thin peptidoglycan cell wall. In addition, an outer membrane that is essential for the survival of *E. coli*, also serves as a protective barrier [20]. *S. aureus* is Gram positive bacteria that are frequently resistant to most currently available antibacterial agents which are extremely difficult to treat [21]. Antibacterial agents may be rendered inactive by three major mechanisms of resistance in Gram-positive bacteria: destruction or modification of the antibiotic (e.g. production of beta-lactamases and aminoglycoside-inactivating enzymes), prevention of access to the target (e.g. alteration of permeability or efflux) and alteration of the target site [21]. It is important to note from the results obtained in Fig. 4, SZB2 was found to inhibit the growth of E. *coli* and *S. aureus*, implying its good potential application to produce wide spectrum antibiotic effective for both Gram positive and Gram negative bacteria.

The 16S rDNA sequence analysis was particularly useful for the confirmation of the SZB2 identity. Genes of SZB2 was sequenced and compared with the sequences deposited in the database following amplification by PCR [10]. What is interesting in this sequence analysis data is that isolated SZB2 was closely related to *Stenotrophomonas maltophilia* with 99% homology. Taken together, the phylogenetic tree (Fig. 5) suggests that there is an association between isolated SZB2 with *Stenotrophomonas maltophilia* species such as AM3-1. The strain was then designated

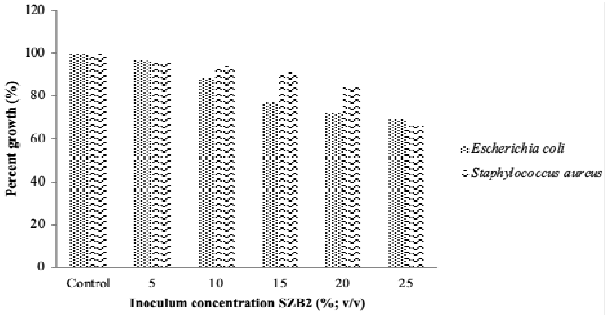

Fig. 4 Antibacterial activity analysis of SZB2.

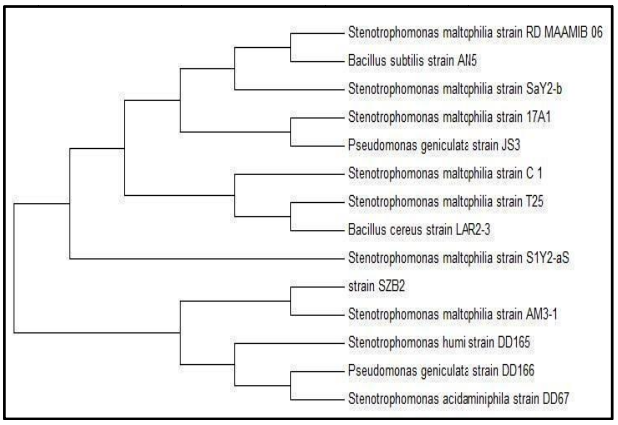

Fig. 5 Phylogenetic tree for SZB2 strain.
Neighbour-joining method was used to construct the tree.

as *Stenotrophomonas* sp. SZB2. As mentioned in the previous study, *Stenotrophomonas maltophilia* is a Gram negative purple photosynthetic bacteria [22].

4. Conclusion

One of the significant findings from this study was that SZB2 showed good potential application for the production of new broad spectrum antibiotic effective to both Gram positive and Gram negative bacteria. SZB2 could be classified as psychrotroph that requires minimum energy usage for its application in the industry. In this study, identification of SZB2 was closely aligned with the genus *Stenotrophomonas* using the 16S rDNA analysis, thus it was designated as *Stenotrophomonas* sp. SZB2. Further research into the antibacterial activity of SZB2 is strongly recommended. In the future, intensive research can be carried out in the following areas: identification of the antibacterial compound after extraction and purification using GC-MS (gas chromatography-mass spectrometry), LC-MS (liquid chromatography-mass spectrometry) or NMR (nuclear magnetic resonance spectrophotometry). Further studies can be explored on the detection of antibacterial genes in SZB2. This could be done by using PCR for amplification of the DNA of SZB2. More information on the antibacterial activity of SZB2 will help establish a greater understanding of its function as a wide spectrum antibacterial compound.

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